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The Mouse *Hoxd13*^{spdh} Mutation, a Polyalanine Expansion Similar to Human Type II Synpolydactyly (SPD), Disrupts the Function but Not the Expression of Other *Hoxd* Genes

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Polyalanine expansion in the human *HOXD13* gene induces synpolydactyly (SPD), an inherited congenital limb malformation. A mouse model was isolated, which showed a spontaneous alanine expansion due to a 21-bp duplication at the corresponding place in the mouse gene. This mutation (synpolydactyly homolog, *spdh*), when homozygous, causes malformations in mice similar to those seen in affected human patients. We have studied the genetics of this condition, by using several engineered *Hoxd* alleles, as well as by looking at the expression of *Hox* and other marker genes. We show that the mutated SPDH protein induces a gain-of-function phenotype, likely by behaving as a dominant negative over other *Hox* genes. The mutation, however, seems to act independently from *Hoxa13* and doesn't appear to affect *Hox* gene expression, except for a slight reduction of the HOXD13 protein itself. Developmental studies indicate that the morphological effect is mostly due to a severe retardation in the growth and ossification of the bony elements, in agreement with a general impairment in the function of posterior *Hoxd* genes.

INTRODUCTION

Vertebrate *Hox* genes are necessary to properly pattern the developing rostro-caudal axis, both in neuro-ectoderm and mesoderm derivatives (e.g., Krumlauf, 1994). For example, specific combinations of HOX proteins found at one particular rostro-caudal level during trunk development will shape the morphology of the corresponding vertebra. While *Hox* gene targets are still largely unknown, experiments in which gene doses were sequentially removed have shown that quantitative as well as qualitative parameters are involved in the cooperation between these genes (e.g., Horan *et al.*, 1995; Gérard *et al.*, 1997; Zakany *et al.*, 1997). This was further demonstrated by exchanging *in vivo* paralogous genes for one another (Greer *et al.*, 2000).

In addition to this crucial contribution to the development of the rostral to caudal axis, tetrapod *Hox* genes within both the *HoxD* and *HoxA* clusters (from group 9 to group 13) are also required for normal appendicular development (Dollé *et al.*, 1989; Haack and Gruss; 1993; Zakany and Duboule, 1999). In limbs, however, the function of these genes seems to be slightly different from their function in the trunk. Combined loss of *Hox* functions in the limbs leads to the disappearance of the corresponding structures, rather than to their transformation into another type of morphology, as occurs in the trunk (Dollé *et al.*, 1993; see Rijli and Chambon, 1997). For example, mice mutant for both group *Hoxa11* and *Hoxd11* genes essentially lack forearms (Davis *et al.*, 1995), whereas mice without *Hoxa13* and *Hoxd13* functions no longer develop any digits (Fromental-Ramain *et al.*, 1996; Kondo *et al.*, 1997). While these observations have been instrumental in our understanding of limb development, they have also provided some information regarding the molecular aetiology of human genetic conditions, which involve mutations within these genes. Indeed, a series of human congenital syndromes exist, which display alteration in the shape

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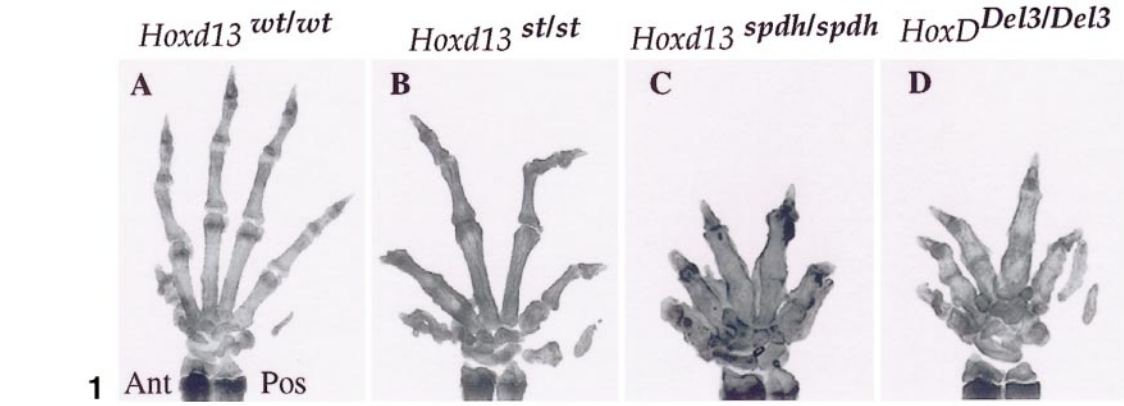


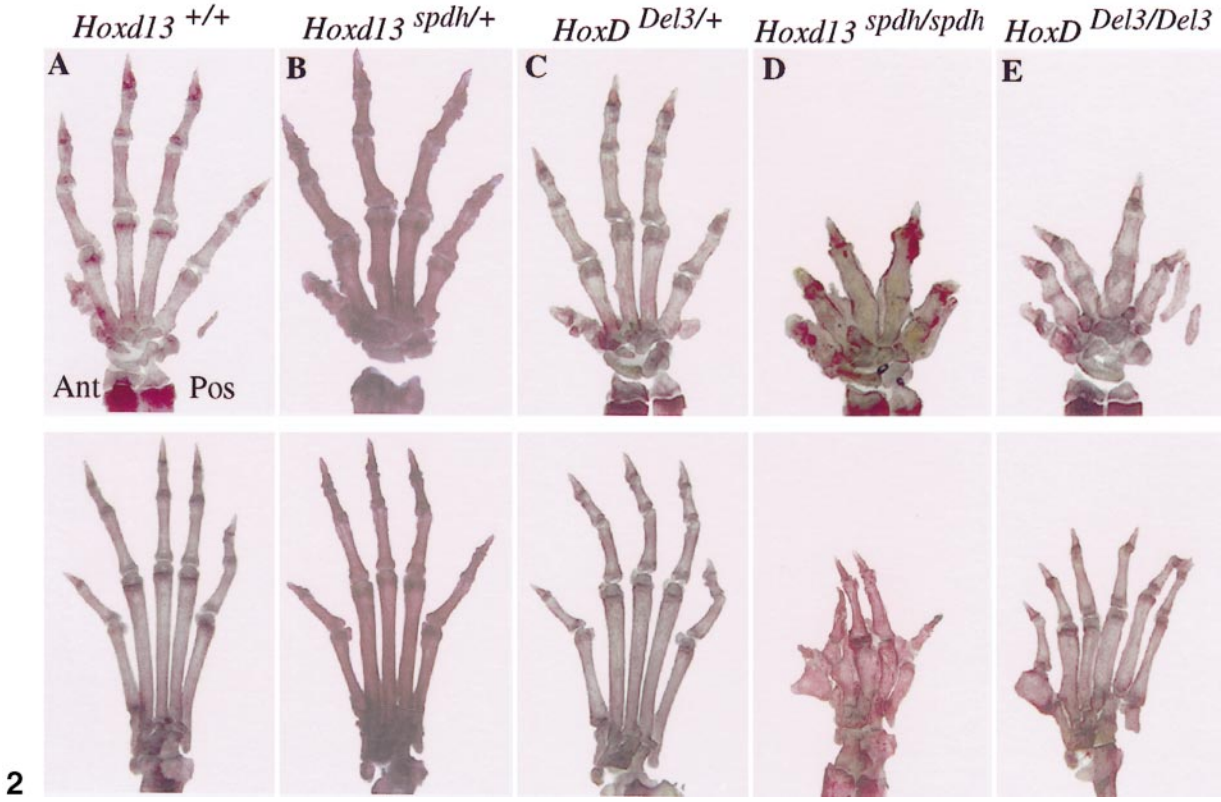


FIG. 1. The *spdh* mutation. Preparations of adult distal forelimb skeletons stained with alizarin red. (A) Control autopod. (B) Distal limb skeleton of a mouse homozygous for the loss-of-function, insertional allele of *Hoxd13* (*Hoxd13*st). A mild defective phenotype was observed, with a slight reduction (ectrodactyly) in the length of digits II and V, due to the absence of their second phalanges. (C) A similar staining for a mouse homozygous for the *spdh* mutation. The alterations were much more pronounced, including an increased ectrodactyly and a rougher and stiffer aspect of the digits. (D) The phenotype obtained for a deficiency in the *HoxD* complex, which inactivated the functions of *Hoxd13*, *Hoxd12*, and *Hoxd11* (*HoxD*^{del3}), was comparable to the homozygous *spdh* phenotype, with about the same degree of digit reduction and a similar ill-formed aspect of the entire bony elements. Ant, anterior; Pos, posterior.

FIG. 2. Comparison between the *spdh* and *Del3* digit phenotypes. (A) Control forelimb (top) and hind-limb (bottom) autopods. (B) Skeletal pattern of a specimen heterozygous for the *spdh* mutation. A subtle reduction in the length of the second phalanges in digits II and V was occasionally scored (30% of the cases). (C) In heterozygous *Del3* animals, however, digit V was always affected, as well as digit II, to some degree, in particular in the forearms (top). (D) Animals homozygous for the *spdh* phenotype showed, by comparison, severe alterations, with a marked ectrodactyly and abnormal aspect of the digits, in both fore- and hindlimbs. Polydactyly was sometimes scored in hindlimbs, though not with a full penetrance. (E) The phenotype of *Del3* homozygous animals was similar to *spdh* homozygotes, except that a polydactylous hindlimb was observed in most, if not all, of the cases. The abnormally flattened and extruding piece of bone, visible on digit I of the *spdh* and *Del3* homozygous hindlimbs, is strongly associated with a loss-of-function of *Hoxd13* (Dollé *et al.*, 1993). This demonstrated that the *spdh* mutation involved at least a loss-of-function of the *HoxD13* protein, even though the protein was present (see below). Ant, anterior; Pos, posterior.

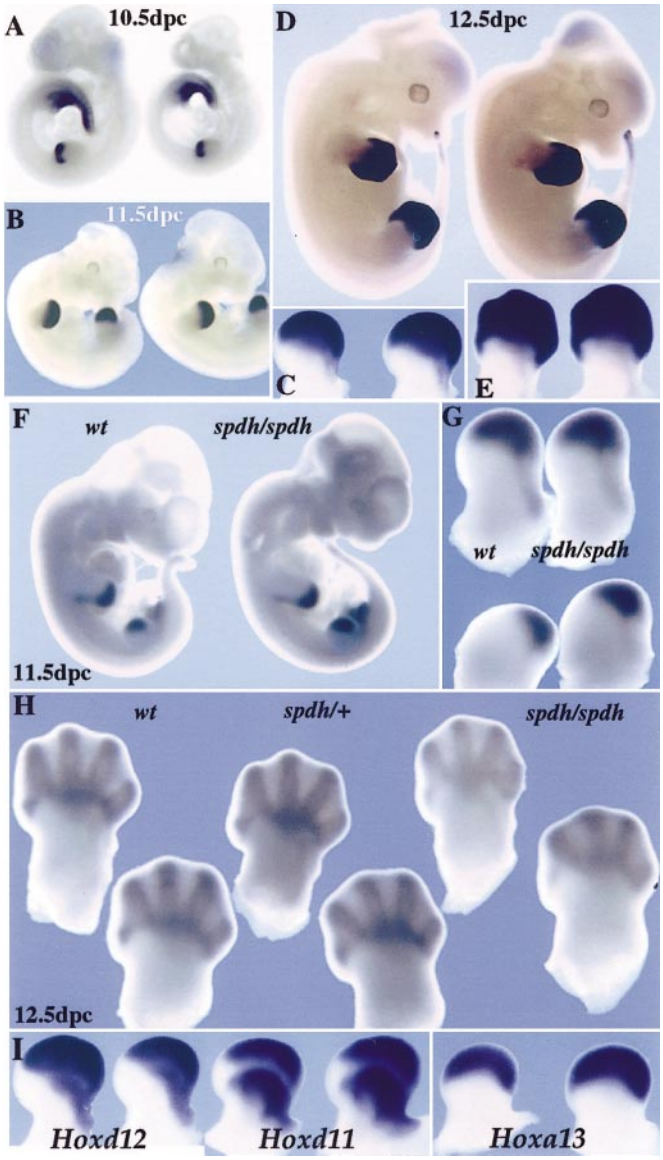


FIG. 3. Expression of posterior *Hox* genes during the development of *spdh* mutant limbs. In all panels, wild-type littermates (left) are shown next to the *spdh* homozygous mutants (right). Expression of *Hoxd13* RNA at 10.5 (A), 11.5 (B), and 12.5 dpc (D) was essentially indistinguishable between control and mutant specimen. At 11.5 (C) and 12.5 dpc (E), closer views of the right forelimb autopods also failed to reveal any clear difference. (F–H**)** Immunodetection of HoxD13 protein, using a polyclonal antibody was performed on either 11.5 (F, G) or 12.5 dpc embryos (E). The presence of the HoxD13 protein was detected at the expected sites (F), in the distal region of the autopods in both fore- (G, top) and hindlimbs (G, bottom). (H) Higher magnifications of 12.5-dpc fore- (top) and hindlimbs (bottom) of control (left), heterozygous (middle), and homozygous (right) *spdh* mutant animals. A weak but significant decrease of HoxD13 signal was observed in homozygous mutant limbs. (I) Expression of the *Hoxd12*, *Hoxd11*, and *Hoxa13* genes in 11.5-dpc homozygous mutant limbs (right). Expression profiles were indistinguishable from control limbs (left). In all dissected limbs, anterior is left and posterior is right.

and/or number of digits and which are caused by modifications of either *Hoxd* or *Hoxa* gene functions (reviewed in Goodman and Scambler, 2001). Consequently, our genetic approach using the house mouse as a model system may shed light on those mechanisms that are disturbed in such human genetic conditions.

This genetic approach has been particularly well illustrated, either with the type II synpolydactyly (SPD), or with the hand–foot–genital syndrome, two human syndromes caused by mutations in the *HOXD13* (Muragaki *et al.*, 1996; Akarsu *et al.*, 1996) and *HOXA13* (Mortlock *et al.*, 1997; Goodman *et al.*, 2000) genes, respectively. In both cases, these mutations are dominant and produce severe phenotypic alterations, suggesting mechanisms involving gain-of-functions. Other mutations within the human *HOXD13* gene, however, have been reported to generate more subtle phenotypes in heterozygotes and may thus be associated with loss-of-functions (Goodman *et al.*, 1998). In the case of SPD, the observed alterations primarily affect the digits, with a variable degree of ectrodactyly as well as a polydactyly and lateral fusions of bony elements (syndactyly), in the proximal part. In addition, bony elements appear somewhat stiffer. The SPD condition was reported to be caused by an expansion of a polyalanine tract within the protein coding region of the HOXD13 protein (Muragaki *et al.*, 1996; Akarsu *et al.*, 1996). Interestingly, comparison between this phenotype and those alterations induced by engineered alleles of the corresponding murine *Hox* genes, revealed that the most resembling phenotype was produced by a multiple inactivation of several genes in *cis*, including *Hoxd13* (Zakany and Duboule, 1996). The fact that a multiple loss-of-function of several murine neighboring genes phenocopied a heterozygous condition for a single of these genes in human strongly suggested that the causative factor of the human SPD condition was the production of a dominant-negative protein. In this view, such a modified protein would affect the function of all *Hoxd* genes involved in digit development (Zakany and Duboule, 1996). Alternatively, this apparent paradox between the phenotypes in mouse and human may reflect fundamental differences in the way these genes are used during ontogeny.

Recently, a spontaneous mutation of *Hoxd13* was isolated in mice, which displayed a polyalanine expansion at the same position as occurs in the human SPD syndrome, hence it was called synpolydactyly homolog, *spdh* (Johnson *et al.*, 1998). Interestingly, however, mice heterozygous for this mutation appeared much less affected than their human counterparts. When both copies of the mutated locus were present, the phenotype was nevertheless similar to human SPD (Johnson *et al.*, 1998). This suggested that, while the general effect of the mutation was conserved between these two species, some variation may exist in the quantitative interplay between these proteins, leading to slight differences in the expressivity of the phenotype. The existence of this mutation in mice allowed us to better characterize its genetic interaction with other *Hoxd* genes,

as well as to further investigate the apparent gain of function mechanism behind the phenotype.

Here, we report a genetic analysis of this mouse mutation using a series of complementation tests, which revealed that the *spdh* mutation does indeed interact with the function of other *Hoxd* genes, besides *Hoxd13*. Additional analyses using marker genes also revealed that the severity of the phenotype scored in *spdh* homozygous mice is likely due to a substantial delay in the ossification of the limb bony elements. This phenomenon had already been described in the case of *Hoxd13* and other *Hoxd* genes, but to a much lower extent (Dollé *et al.*, 1993; see Rijli and Chambon, 1997). Therefore, the phenotype induced by the abnormal HOXD13 protein can be best explained by its negative impact on other *Hoxd* genes. No evidence could be found for a specific effect of the mutant protein in inducing cell death, an effect that could have lead to a similar phenotype.

MATERIALS AND METHODS

Alleles and Genotyping

The *spdh* strain (B6C3Fe-*a/a-Hoxd13*^{spdh}) was provided by the Mouse Mutant Resource Colony of The Jackson Laboratory (Bar Harbor, ME). All animals and embryos were genotyped, either by PCR for the *spdh* allele or by Southern blot analysis for the *Del3* (Zakany and Duboule, 1996) and *st* (Dollé *et al.*, 1993) alleles. The PCRs were carried out as previously described (Johnson *et al.*, 1998), with an additional 16% of DMSO. Amplification consisted of one cycle of denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 20 s, and extension at 72°C for 15 s.

In Situ Hybridizations, Immunodetections, Histology, and Skeletal Preparations

In situ hybridizations were performed as described (Gérard *et al.*, 1996), with conventional probes (*Hoxd13*, Dollé *et al.*, 1993; *Hoxd12*, Izpisua-Belmonte *et al.*, 1991; *Hoxd11*, Gérard *et al.*, 1996; *Hoxa13*, gift of F. Rijli). Immunodetections were performed on cryosections, using the anti-HoxD13 antibody at a dilution of 1/100. For skeletal preparations, adult or 8-dpp animals were sacrificed, dissected, eviscerated, and stained according to standard Alizarin and Alcian blue staining protocols (Inouye, 1976).

RESULTS AND DISCUSSION

Phenotypic Analysis

The phenotype induced by a conventional loss-of-function of *Hoxd13* has been previously described (Dollé *et al.*, 1993; Davis and Capecchi, 1996). It was characterized by a severe ectrodactyly of digits II and V, due to the absence of their second phalanges, and a series of alterations in both the shape and number of metacarpal and carpal bones. Furthermore, in about half of the cases, an additional postaxial (posterior) digit was observed near digit V (Figs. 1A and 1B; Dollé *et al.*, 1993; Davis and Capecchi, 1996). Interestingly, the phenotype observed in distal limbs of *spdh* homozygous mutant animals was much more severe. In particular, the ectrodactyly was more pronounced and it was combined with both lateral fusions (syndactyly) and duplications of digits (polydactyly; Fig. 1C; Johnson *et al.*, 1998). In fact, the *spdh* phenotype much more resembled the condition recorded for mice carrying a triple inactivation in *cis* of *Hoxd13*, *Hoxd12*, and *Hoxd11* (*HoxD*^{del3} or *Del3*; Fig. 1D; Zakany and Duboule, 1996). This similarity suggested that the *spdh* mutation did not result in a mere inactivation of *Hoxd13* function, but instead may have induced an interference with the functions of neighboring *Hoxd* genes leading to this appearance of a combined phenotypic effect.

A detailed comparison between the *spdh* phenotype and that of mice lacking the three posterior gene functions (*Hoxd13*, *Hoxd12*, and *Hoxd11*; *Del3*), revealed important differences. While *Del3* heterozygous mice displayed a clear and penetrant alteration in digits, with a visible reduction in the size of the second phalange of digits II and V (Fig. 2C), *spdh* heterozygous animals did not consistently show such an alteration (Fig. 2B). Indeed, only 30% of *spdh* heterozygotes showed a slight reduction in the size of these phalanges, and a fusion of carpals bones was seen occasionally. The mutation was therefore considered only partially dominant, with a low penetrance. However, the phenotype of mice homozygous for *spdh* was very similar to, if not more severe than, that of *Del3* animals (Figs. 2D and 2E), leading to an apparent paradox in the strengths of the phenotypic alterations. This was particularly visible with the feet of *spdh* mutant animals, which were clearly more affected than those of *Del3* (Figs. 2D and 2E, bottom), with a much more severe ectrodactyly of the metatarsal bones, which were all ill-shaped. Interestingly, *spdh/spdh* animals displayed in their hindlimbs an alteration typical of *Hoxd13* loss-of-function, a flattened and extruding piece of bone on digit I (Dollé *et al.*, 1993), indicating that *Hoxd13* function was indeed lost in this condition, even though the protein was present (see below). These observations suggested that *spdh* might interfere with the functions of more *Hox* genes than the three deleted in the *Del3* mutation, either within the *HoxD* (*Hoxd10*) or *HoxA* (*Hoxa13*) complex. In order to investigate whether the negative effect of *spdh* may act at the transcriptional level to lead to the misexpression of one or several *Hox* genes, we looked at the expression of *Hox* genes during limb development in the various genotypes.

Hox Gene Expression in spdh Mutant Limbs

Upon visual inspection, the homozygous *spdh* phenotype was readily visible at 12.5 dpc, at the time when digit prechondrogenic condensations start to appear and to prefigure the future bone pattern. A general, slight reduction in the size of developing forelimbs was detected, as well as a clear delay in the formation of bone models. The time of activation of *Hoxd13* was not significantly different in *spdh* mutants as compared to control littermates (i.e., ca. 15

somites; not shown). *Hoxd13* expression in *spdh* mutant limbs was as in wild-type animals, also in subsequent stages, from 10.5 to 12.5 dpc (Figs. 3A–3E). This clearly indicated that the *spdh* mutation did not affect the transcription of *Hoxd13* itself. To further analyze whether *spdh* might affect the translational control of *Hoxd13*, we looked at the protein distribution by whole-mount immunochemistry using a polyclonal antibody (Figs. 3F–3H). In wild-type littermates, the HoxD13 protein was detected in a slightly more restricted domain, at the distal aspect of the autopod mesenchyme, when compared with the *Hoxd13* mRNAs. As expected, the protein was excluded from the overlying ectoderm. Here again, the HoxD13 protein distribution was similar between *spdh* mutants and control littermates, with respect to both space and time in developing distal autopods (Figs. 3F and 3G). In older limbs, however, the polyclonal antibody repeatedly revealed a slight, but significant, decrease in the amount of protein in homozygous mutant animals, when compared to both heterozygous and wild-type littermates (Fig. 3H). While this may reflect either a genuine lower amount of the protein, or the loss of reactivity with the antibody due to the mutation of the protein, such a mild decrease couldn't account for the observed phenotype. Together, these results indicated that a modified HoxD13 protein was indeed produced by the *spdh* mutant locus, perhaps in slightly lower amount, and that its distribution was as for its wild-type counterpart. This was consistent with the view whereby a dominant-negative protein was the cause of the phenotype.

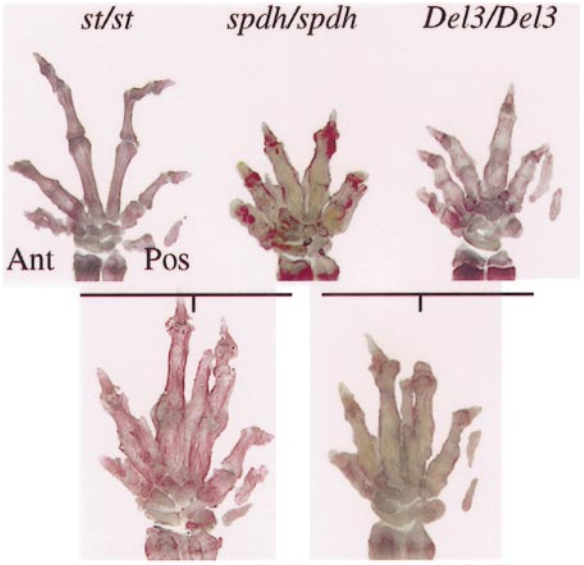
As the existence of auto- and/or cross-regulatory interactions between vertebrate *Hox* genes and their products has been documented in different contexts (e.g., Zappavigna *et al.*, 1991; Studer *et al.*, 1998), we looked at the effect of the *spdh* mutation on other *Hox* genes involved in digit development. To assess whether the modified HoxD13 protein was modifying in some way the expression of other posterior *Hox* genes involved in the development of the autopod, we analyzed the expression of *Hoxd12*, *Hoxd11*, and *Hoxa13* by whole-mount *in situ* hybridization with antisense riboprobes (Fig. 3I). None of these genes were found affected by the *spdh* mutation (Fig. 3I). This result suggested that the *spdh* mutation did not interfere with the transcription of those posterior *Hox* genes necessary for autopod development. Consequently, a dominant-negative effect, if any, would not involve the transcriptional down-regulation of either *Hoxd* or *Hoxa* genes, but would more likely occur through functional interference, such as competition between an inactive HoxD13 protein and other HoxD proteins for binding sites, or titration of co-factors. Therefore, we next analyzed whether the mutation would affect the functional activity of these posterior genes, rather than their transcription, by using a genetic approach.

Genetic Analysis

We carried out complementation tests, by crossing *spdh* mice with mice carrying either the above described triple inactivation in *cis* of *Hoxd13*, *Hoxd12*, and *Hoxd11* (*Del3;* Zakany and Duboule, 1996), or an insertional null allele of *Hoxd13* (*Hoxdst*; Dollé *et al.*, 1993). Mice trans-heterozygous for both *spdh* and the *Hoxd13* loss-of-function allele (*Hoxd13$^{spdh/st}$*) displayed digit phenotypic alterations somewhat intermediate between that present in mice homozygous either for the *st* or the *spdh* alleles (Fig. 4; cross on the left). This result genetically demonstrated that *spdh* was not a mere inactivation of *Hoxd13*, in which case the *st/st* phenotype would have been observed. Furthermore, trans-heterozygous *spdh/st* animals resembled those carrying a double inactivation of *Hoxd12* and *Hoxd13* (Kondo *et al.*, 1998), including the full disappearance of the second phalanges in digits II and V, as well as a thickening of digits and longitudinal fusion of phalanges in digits III and IV. This further suggested that the *spdh* mutation had interfered with the function of *Hoxd12* in addition to that of *Hoxd13*.

As expected, *Del3/spdh* trans-heterozygous mice displayed a more severe phenotype than that seen in *st/spdh* trans-heterozygous animals, mostly due to a stronger ectrodactyly, though the number and arrangement of the bony elements were quite comparable in both cases (Fig. 4; compare both panels at the bottom). Surprisingly, however, the phenotype of the *Del3/spdh* trans-heterozygous animals was clearly less severe than that displayed by mice homozygous for either one of the parental alleles, suggesting that a partial complementation between *Del3* and *spdh* had occurred (Fig. 4). This result can be explained by the fact that a single dose of *spdh* may not be sufficient to functionally inactivate the remaining doses of *Hoxd12* and *Hoxd13*, as suggested by the very weak phenotype recorded in *spdh* heterozygous animals. In this view, trans-heterozygous animals would still be able to exert some *Hoxd12* and *Hoxd11* function; hence they would be less affected than either one of the homozygous parental alleles. The observation that the *Del3/spdh* phenotype was more severe than *Del3/st* (Fig. 4; bottom) confirmed the proposal that *spdh* was able to alter the activity of at least *Hoxd12* and *Hoxd11*, in addition to its own. The sole difference between *Del3/spdh* and *st/spdh* was the presence of an additional copy of *Hoxd12* and *Hoxd11* in *st/spdh*. Strikingly, both trans-heterozygous animals exhibited a feature previously described for human SPD, consisting of a variable syndactyly of digits III and IV, along with a supernumerary digit in between (Muragaki *et al.*, 1996). Moreover a partial complementation between these alleles was also observed in the reproductive performance, as about 60% of the males were fertile, whereas both homozygous parental conditions were sterile (Dollé *et al.*, 1993; Johnson *et al.*, 1998). Taken together, these results showed that two doses of the *spdh* alleles were necessary for a full dominant-negative effect. It also raised the possibility that this dominant-negative activity would affect *Hox* genes other than the neighboring *Hoxd11* and *Hoxd12*.

As the only other *Hox* gene required for digit development is the most posterior member of the *HoxA* complex, *Hoxa13* (Haack and Gruss, 1993), we introduced a loss-of-



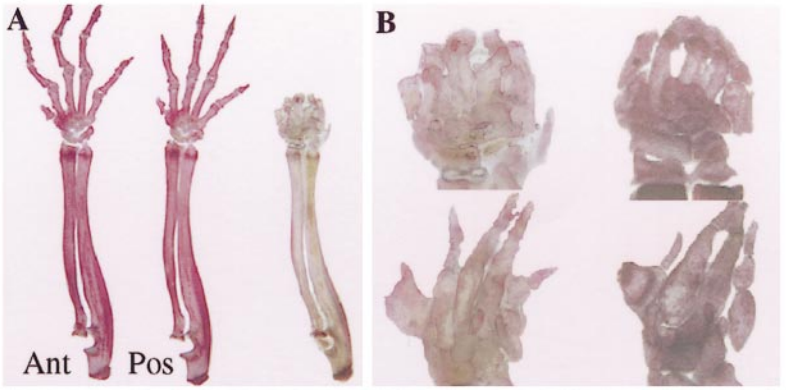


FIG. 4. Complementation tests between *spdh* and other *HoxD* alleles. On the top, the phenotypes of the three homozygous alleles used for the complementation tests. On the bottom panels, adult forelimb skeletal preparations of trans-heterozygous animals derived from the crosses between *spdh* mice and mice with the two other alleles. Interestingly, the cross between mice with the *spdh* and the *Del3* alleles generated an F_1 phenotype that was weaker than either one of the two parental alleles when homozygous.

FIG. 5. Genetic interaction between *spdh* and *Hoxa13*. (A) When one dose of *Hoxa13* was removed from the *spdh* heterozygous background (middle), the observed effect on digit morphology was readily visible and stronger than one dose of *spdh* alone (left). When one dose of *Hoxa13* was removed from a *spdh* homozygous background, the effect was drastic, generating a completely truncated autopod with barely recognizable ectrodactylous digits, fused laterally to each other (right), resembling the alterations produced by a $Hoxd13^{st/st}$; $Hoxa13^{+/-}$ double condition. (B) When one dose of *Hoxa13* was removed from a background homozygous for either the *spdh* mutation (left) or for *Del3* (right), the resulting phenotype was almost identical, in both forelimbs (top) and hindlimbs (bottom). These results suggested that the *Hoxa13* allele did not specifically interact with the *spdh* mutation, or that the dose of *spdh* protein was not high enough to exert its negative effect over the *Hoxa13* function. Ant, anterior; Pos, posterior.

function allele of this latter gene in the genetic configuration analyzed above, to unravel potential interactions. This loss-of-function allele, however, is homozygous lethal (Fromental-Ramain *et al.*, 1996), hence only animals carrying a single copy of it could be assessed. We first combined one or two doses of *spdh* with the *Hoxa13* mutation. The loss of one dose of *Hoxa13* slightly affected the formation of digit I (Fig. 5A). However, when one copy of *spdh* was added, a clear ectrodactyly appeared, due to the absence of second phalanges in digits II and V (Fig. 5A; middle). This

phenotype was similar to the *Del3* heterozygous condition, indicating that, in a *Hoxa13* heterozygous background, the dominant-negative effect of *spdh* was enhanced. This non-allelic noncomplementation illustrated the necessary cooperation between *Hoxa* and *Hoxd* gene functions, to properly build up the digits. The addition of another dose of *spdh* over the *Hoxa13* heterozygous background, generated a drastic reduction in the size of all digits, as well as a general syndactyly (Fig. 5A; right), comparable to the phenotype observed in *Hoxa13*$^{+/-}$; *HoxD*$^{Del3/Del3}$ double mutants (Fig. 5B). This phenotype was nevertheless not as severe as a complete loss-of-function of *Hoxa13*, which was characterized by a digit agenesis (Fromental-Ramain *et al.*, 1996; Kondo *et al.*, 1997). Therefore, these results suggested that *spdh* did not directly interfere with *Hoxa13* function. However, we cannot rule out the possibility that additional doses of the *spdh* protein may have a stronger effect on *Hoxa13* function, which might occur if the amount of mutated *Hoxd13* protein is a limiting factor in the dominant-negative effect. Experiments involving the massive expression of a transgenic mutated *spdh* protein in developing limbs should be informative in this respect.

Effect of the spdh Mutation on the Ossification Process

Previous studies on the function of *Hoxd* genes during chondrogenesis and the establishment of the bone models have revealed that these genes are transcriptionally active in chondrogenic cells of the growth plate, whereas they are switched off at the time cells become hypertrophic (Zakany and Duboule, 1996). We assessed the effect of the *spdh* mutation on the endochondral ossification process by comparing *spdh* homozygous mutant limbs with various trans-heterozygous conditions. Eight days after birth (8 dpp), ossification centers were observed both in *spdh/st* and *spdh/Del3* trans-heterozygous feet, whereas they were completely absent from *spdh* mutant limbs (Fig. 6A). This enhanced delay in the ossification process, observed in *spdh* homozygous mutants when compared to trans-heterozygous conditions, was reminiscent of that observed in *Del3* mutants (Zakany and Duboule, 1996). Therefore, this observation further confirmed that the *spdh* phenotype was established primarily through a general functional inactivation of *Hoxd* genes, in a dose-dependent fashion. A severe delay was observed in the transition of cells from the chondrogenic to the hypertrophic stage, leading to the absence of calcification at the time when this process was already well under way in control specimens.

To see when the first signs of this alteration could be observed, we looked at an early marker of chondrocyte formation, collagen type II (*Col2*), which labels the pattern of prechondrogenic condensations. In 12.5-dpc wild-type autopods, a robust *Col2* expression was detected, which prefigured the future organization of bony elements (Fig. 6B). Interestingly, similarly aged *spdh* limbs showed a significantly fainter staining (Fig. 6B). In addition, the bone models were not properly spaced and distributed. In particular, the occurrence of an ectopic bone model was observed, likely induced by a too large distance between two such condensations (Fig. 6B). This abnormal *Col2* staining pattern prefigured the supernumerary bone routinely observed

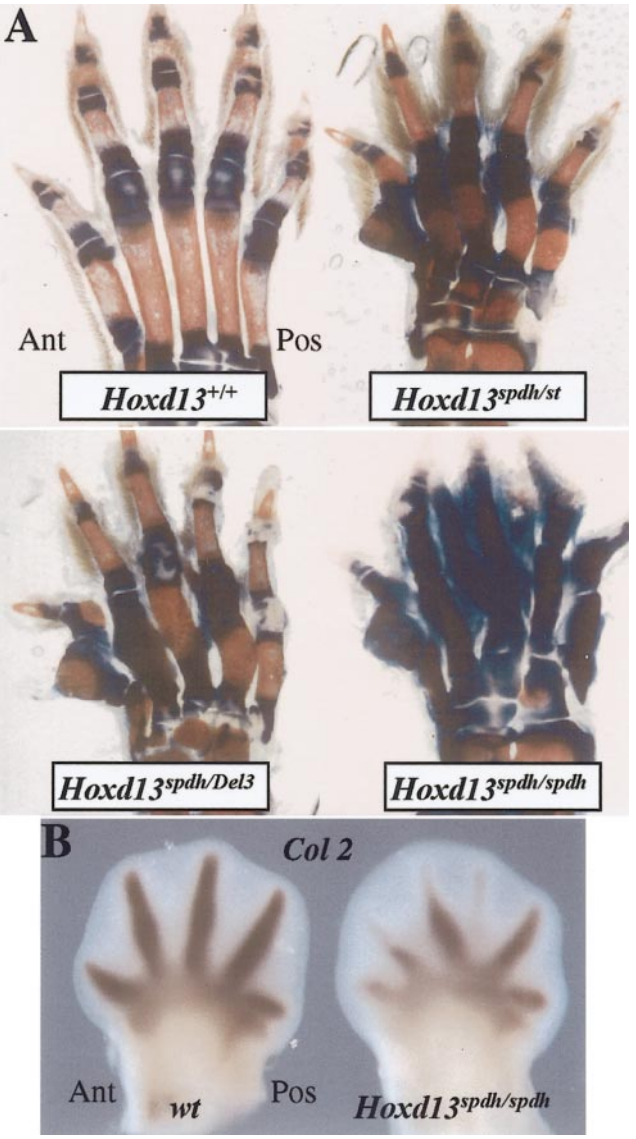


FIG. 6. Effect of the *spdh* mutation on endochondral ossification. (A) Skeletal preparations of specimens eight days after birth. Both *spdh/st* and *spdh/Del3* limbs displayed some delay in the ossification process, when compared with the control animal (top left). This delay in the appearance of alizarin stained material was strongly enhanced in *spdh* homozygous animals, as no bone formation could be observed at a similar stage (bottom right). (B) In 12.5-dpc fetal limbs, the expression of the collagen type II (*Col2*) gene in *spdh* mutants (right) was markedly different from wild type, already reflecting the disorganized pattern of the bony elements, the severe ectrodactyly as well as the occurrence of supernumerary condensations. Ant, anterior; Pos, posterior.

in adult *spdh* animals (Fig. 6A). This result indicated that the delay in ossification, characteristic of *spdh* mutants, was established early on during limb development, in the course of primary chondrogenesis. This supports a view whereby *Hoxd* gene function is necessary both to properly organize the early pattern of prechondrogenic condensations, as well as to trigger cells to progress from an early state of chondrogenic differentiation toward hypertrophic cartilage cells.

Finally, and even though the above results did not support such a possibility, we looked whether the phenotype induced by the *spdh* mutation could be associated with an enhanced cell death, a mechanism described to occur in cases where proteins show large expansions of stretches of monotonic residues. Using an anti-caspase-3 antibody, we were not able to detect any difference between wild-type and *spdh* mutant limbs, indicating that cell death is not a major factor responsible for the phenotypic alterations observed in this mutant strain (not shown). Together, our data suggest that the triplet expansion in the mouse HoxD13 protein leads to a protein that not only lost its functional properties, but also antagonized the functions of other *Hox* genes. The *spdh*-associated loss of *Hoxd13* function may rely upon changes, either in the protein conformation, or in the modification of an important protein domain. The interference with other, but related, *Hox* gene functions may involve the binding of the mutant protein to sites that otherwise (in the complete absence of *Hoxd13*) would be occupied by other Hox proteins. Alternatively, the mutated protein could sequester some cofactors necessary for other Hox proteins to correctly achieve their functions. A transgenic system allowing for the expression of various forms of the HoxD13 protein in limbs should help to discriminate between these alternatives.

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